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Toxicologic Screening of Agmatine Using *In Vitro* and *In Vivo* Assays

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Objective To evaluate the both *in vitro* and *in vivo* toxicological effects of agmatine.

Methods The toxicity of agmatine (50, 100 and 200 μM) was determined by using Alamar Blue assay in murine brain endothelial (bEnd.3) cells as *vitro* model and chick chorioallantoic membrane (CAM) assay as an *in vivo* model.

Results The treatment of agmatine (50, 100 and 200 μM) showed no significant increase/decrease in the total cell number compared to control in the bEnd.3 cells in *in vitro*. The treatment of agmatine at three different concentrations (50, 100 and 200 μM) onto the CAM tissue for 2 days, showed similar survival rates of chick embryo to that of the control group.

Conclusions The present results demonstrated that agmatine (50, 100 and 200 μM) doesn't exert any toxic effect as evidenced by the above results depicting the therapeutic potential of agmatine via modulation of vascularization for the treatment of neural diseases.

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Key Words Agmatine, Toxicology, Endothelial cell, Chick chorioallantoic membrane, Neurovascular unit.

Introduction

Even interruption of energy or oxygen for a moment, can lead to critical impact on survival of brain. Brain relies on a constant perfusion to fulfill its energetic demands, in particular during increased neuronal activity.[1] Circulation of blood has to deliver oxygen, glucose and other nutrients from blood to brain, while metabolites (ex. Lactic acid and carbon dioxide) must be removed. There is a tight relation between cerebral blood flow and neuronal activity, named neurovascular unit.[2,3] The complex interplay between neurons (terminals from the glutamatergic pyramidal cells and gamma-aminobutyric acid interneurons), astrocytes endfeet and blood vessels (endothelial cells, myocytes and pericytes) forms the 'neurovascular unit' which supply firstly blood to areas of neuronal activation.[4,5] Neuroimaging studies using functional magnetic resonance imaging and positron emission tomography also give appropriate help to confirm the exitence of these mechanisms.[6] Recently, the focus on therapy of neural diseases has shifted from neuronal treatment itself to vascular treatment. Furthermore, neurovascular unit has been magnified as target developing new drug for treatment of neural disease.

Agmatine, a polycationic amine, is synthesized by decarboxylation of L-arginine by arginine decarboxylase (ADC). Some researchers have revealed that agmatine has anticonvulsant,[7,8] antineurotoxic,[9-11] and antidepressant actions.[12,13] In our studies, exogenously administered agmatine protects neurons and astrocytes against ischemic injury.[10,14,15] Agmatine seems to modulate various functions in the heart, brain and vasculature as well as a number of effects on calcium homeostasis.[16] Agmatine decreased the expression of matrix metalloproteinase-2 (MMP-2) and MMP-9 expression induced after ischemic injury via the increase of endothelial nitric oxide synthase (eNOS) in the cerebral endothelial cells.[17-19] These results reflect that agmatine has effect on vascular system as well as central nervous system.

Toxicity tests are required as basic step for developing new drug. Live animals are the most reliable subjects for testing medicines for toxicity before commercialisation. Chick chorioallantoic membrane (CAM) assay is considered as one of the model not only for determining angiogenesis at the embryonic stage, but also for toxicological studies.[20] In the present study, we examined the toxicological effects of agmatine for treatment of neural diseases by using the murine brain endothelial (bEnd.3) cells as an *in vitro* experimental model and CAM assay as an *in vivo* model. Our results indicate that agmatine doesn't show toxic effect at all the concentrations of 50, 100 and 200 μM in our present study.

Materials and Methods

Cell culture

Murine brain endothelial cells (bEnd.3 cells, #CRL-2299, ATCC, Manassas, VA, USA) were purchased from ATCC and cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL, Grand Island, NY, USA) supplemented containing 10% (v/v) fetal bovine serum (FBS, Hyclone Laboratories, Logan, UT, USA) and 100 units/mL penicillin/streptomycin (Sigma, St Louis, MO, USA) and were incubated at 37°C in a humidified atmosphere containing 5% CO_2 until they attain the confluency.

Effect of agmatine in *in vitro* model

The *in vitro* toxicity of agmatine on bEnd.3 cells was determined by Alamar Blue assay. Briefly, cells (6×10^3 cells/well) were seeded in 96-well plates, incubated for 24 h in normal media and later the cells were starved for 24 h in media added with 0.1% FBS, and treated without or with various concentrations of agmatine (50, 100 and 200 μM, Sigma, St Louis, MO, USA).

Culture medium was removed from each well and was transferred to the fresh 96-well plate. The Alamar-Blue® assay solution (10 μL; Invitrogen) was added to each well and cells were incubated for 24 h. After the incubation, the developed fluorescence was measured using Spectra Max Gemini EM spectrophotometer (Molecular Devices Cooperation, Sunnyvale, CA, USA) with an excitation wavelength of 570 nm and an emission wavelength of 630 nm ($570_{Ex}/630_{Em}$). Fluorescence quantified was represented as 100% in control medium without agmatine treatment and was displayed as the relative value to that of control.

Effect of agmatine in *in vivo* model

CAM model was accepted for determining the *in vivo* toxicity of agmatine if any.[21] In brief, fertilized brown Leghorn eggs, obtained from Pulmuone Food Co., Seoul, Korea, were kept with the narrow apex down into an egg incubator at 37°C with a relative humidity of 55%. After day 3.5 of incubation, 2.5 mL of albumen was aspirated from the eggs using 3 mL syringe with a sterile 18G needle through the small hole drilled at the blunt apex of the eggs, allowing the small chorioallantoic membrane and yolk sac to drop away from the shell membrane. The small hole was then sealed with parafilm tape (American National Can, Chicago, IL, USA) and eggs were placed in a static mode into the incubator for 1 h. After the removal of parafilm tape, a round window approximately 1.3 cm^2 was cut out by forcep, and the shell membrane on the floor of the air sac was peeled away and covered with cellophane tape. The underlying CAM was accessed through this window. At day 4.5 of incubation, agmatine (50 μM) was loaded on Thermanox plastic coverslip (Nalgene Nunc International, New York, NY, USA) and was applied onto the developing CAM surface. Two days after returning the chick embryo to the incubator, an appropriate volume of 10% fat emulsion (Intralipose®, 10%) was injected into a 6.5-day-old embryo chorioallantois. The eggs were then observed under a microscope. The survival of chick embryo was checked by the naked eye and images of CAM was captured using digital camera.

The survival rate at 2 days post-treatment, was determined by the following formula:

$$\text{Survival (\%)} = A_{\text{alive eggs}} / A_{\text{total eggs}} \times 100$$

$A_{\text{total eggs}}$; the total number of eggs used for experiment.

$A_{\text{alive eggs}}$; the total number of eggs survival after the experiment.

Statistical analysis

All experiments were expressed as mean±S.E.M. Three to five separate experiments were performed for each study. Data were analyzed by Student's t-test or ANOVA test. p value <0.05 were considered statistically significant.

Results

Non-toxic effect of agmatine in *in vitro*

The *in vitro* toxicity of agmatine, the number of live cell was quantified using the Alamar Blue colorimetric assay. The treatment of agmatine (50, 100 and 200 μM) doesn't show significant decrease in the total cell number with the same start of cell seeding number at the time of experiment (6×10^3 cells/well). The percentage of total number of cells at 50, 100 and 200 μM. Concentration of agmatine was recorded as 99.7%, 103.6% and 99.6%. Respectively compared to 100% cells in the control group (Fig. 1).

Non-toxic effect of agmatine in *in vivo*

In vivo toxicity of agmatine was evaluated using the CAM

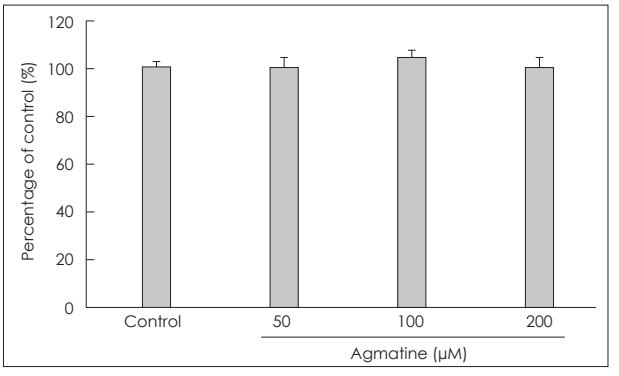

Figure 1. Agmatine did not show any toxicity on bEnd.3 cells *in vitro*. Cells (6×10^3 cells/well) were seeded on 96-well plates, starved for 24 h in 0.1% FBS and were incubated for 24 h without (control) or with increasing concentration of agmatine (0, 50, 100 and 200 μM). The cell number is given as a percentage of controls (100%). Columns represent means±S.E.M of data (n=5). S.E.M: standard of the mean.

Table 1. Survival rate of eggs treated by agmatine of several concentrations

Concentration of agmatine (μM)	Total eggs	Lethal eggs	Survival (%)
Control	31.0±0.6	3.0±0.6	90.4±1.7
50	31.3±0.9	4.3±0.9	86.1±3.0
100	30.7±1.2	3.0±1.0	90.3±3.2
200	31.0±0.6	4.0±1.5	87.2±4.7

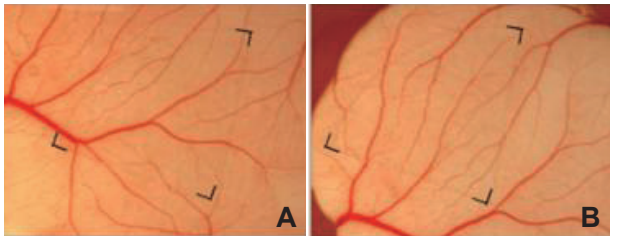

Figure 2. Surface images of the CAM at 2 days following application of vehicle (saline) and agmatine. Micrograph of experimental control group (EC) in which only saline as vehicle was used instead of agmatine and the images were taken after 2 days of incubation of saline (A) and agmatine (50 μM, B) applied onto the CAM. CAM: chick chorioallantoic membrane.

model. After the treatment of 1 μL of agmatine solution containing different concentrations (50, 100 and 200 μM) onto the CAM tissue for 2 days, the chick embryo survival was determined. Table 1 showed that survival rate (86.1%, 90.3% and 87.2%) of chick embryo treated with agmatine (50, 100 and 200 μM) was found to be almost compared to the control group (90.4%). The obtained results depicit that agmatine doesn't exert any toxic effect until 200 μM concentration (Fig. 2).

Discussion

The blood-brain barrier, not only forms a restrictive and protective barrier between blood and the neuronal parenchyma, but also consists of a tightly sealed monolayer of brain endothelial cells that regulate blood flow, nutrient transport, ion homeostasis, clearance of toxic substrates, and various other physiologic processes by the collective action of neurons, astrocytes, pericytes, and microglias.[22,23] This interaction is referred to as the 'neurovascular unit', also named neurovascular coupling. Nowadays, a growing number of studies are paying attention to this concept.[22,24-27] The importance of this concept has been supported by many recent studies showing that common signals and substrates underlie neuronal and vascular biology.[28,29]

Agmatine is a metabolite of arginine synthesized by ADC. Treatment of agmatine was systemically found to enhance the analgesic effect of morphine[30-32] and inhibit tolerance to morphine analgesia.[33] In addition, it also has neuroprotective effects in persistent pain and neuronal injury models[34] suggesting that agmatine may have the pharmaco-therapeutic potential for the treatment of central nervous system (CNS) diseases. Agmatine has been shown to be packaged in synaptic vesicles in the brain and was spinal cord, and released upon depolarization.[35] With several effects on calcium homeostasis, agmatine seems to modulate various functions in the heart, brain and vasculature.[16] We provided evidence that agmatine attenuated expression of MMP-2 and MMP-9 expression which was induced by ischemic injury through the increase of eNOS in the cerebral endothelial cells.[17-19] According to these reports, agmatine may have an effect on neurovascular unit. Moreover, agmatine (agmatine sulfate) is recently available as supplementary health food taking shape of capsule on the website of www.amazon.com. Thus, agmatine has sufficient potential to be developed as new medicine for the treatment of neural diseases.

It was reported that agmatine inhibited proliferation of both human leukemia cell lines which were in the same range as those determined for its inhibition of proliferation human and rat hepatoma cells[36] and mouse kidney proximal tubule cells[37] and which are achievable *in vivo*. The antiproliferative effect of exogenous agmatine was paralleled by cell line-specific changes in agmatine forming and degrading enzymes which were revealed by both qPCR and microarray gene expression data analysis.[38] Inhibition of cell proliferation by agmatine has been shown to be associated with a reduction of the intracellular levels of the polyamines.[37,39] Parralled to these results, there are some results that agmatine usually inhibits proliferation of tumor cell. Our results clearly demonstrate that treatment of agmatine (50, 100 and 200 μM) is found to be not toxic in murine bEnd.3 cells (Fig. 1) and CAM (Fig. 2, Table 1), as evidenced in *in vitro* and *in vivo* test. 'Neurovascular unit' reflects close correlation with blood supply (vessel) and activation of brain (neuronal activity).[2] Our study for the first time provided evidence that agmatine until 200 μM doesn't exert any toxic effect in brain endothelial cells and CAM suggesting that agmatine may have therapeutic potential through the modulation of vascularization against neural diseases.

All together, our *in vitro* and *in vivo* studies showed non-toxic effect of agmatine (50-200 μM), thus suggesting the potential of agmatine for the treatment of CNS diseases.

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